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Steroid-sensitive Gene 1 Is a Novel Cyclic GMP-dependent Protein Kinase I Substrate in Vascular Smooth Muscle Cells*

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Background: Protein kinase GI (PKGI) regulates multiple cardiovascular processes, but its effectors remain incompletely understood.
Results: We identified steroid-sensitive gene 1 (SSG1) as being regulated by PKG in vascular cells.
Conclusion: SSG1 functions as a PKGI kinase target and interacting partner in cardiovascular tissues.
Significance: Identifying new PKGI effectors may improve the understanding of the pathophysiology of cardiovascular diseases.

NO, via its second messenger cGMP, activates protein kinase GI (PKGI) to induce vascular smooth muscle cell relaxation. The mechanisms by which PKGI kinase activity regulates cardiovascular function remain incompletely understood. Therefore, to identify novel protein kinase G substrates in vascular cells, a λ phage coronary artery smooth muscle cell library was constructed and screened for phosphorylation by PKGI. The screen identified steroid-sensitive gene 1 (SSG1), which harbors several predicted PKGI phosphorylation sites. We observed direct and cGMP-regulated interaction between PKGI and SSG1. In cultured vascular smooth muscle cells, both the NO donor *S*-nitrosocysteine and atrial natriuretic peptide induced SSG1 phosphorylation, and mutation of SSG1 at each of the two predicted PKGI phosphorylation sites completely abolished its basal phosphorylation by PKGI. We detected high SSG1 expression in cardiovascular tissues. Finally, we found that activation of PKGI with cGMP regulated SSG1 intracellular distribution.

NO is an important endogenous signaling molecule that induces vascular smooth muscle relaxation and regulates varied cardiovascular processes *in vivo* (1–5). NO primarily stimulates soluble guanylate cyclase in vascular smooth muscle cells (VSMCs)[5] to increase intracellular cGMP production. cGMP, in turn, binds and activates cGMP-dependent protein kinase I (PKGI), the principal cGMP and NO effector in VSMCs (6–8). Although two PKG genes exist (PKGI and PKGII), only PKGI is expressed in cardiovascular tissue (9). The PKGI gene has two splice variant isoforms, Iα and Iβ, which differ only in their amino terminal regulatory domains. The remainder of the PKGI gene encodes autoinhibitory autophosphorylation sites, followed by two cGMP-binding domains, and the carboxyl terminus contains the catalytic domain (10).

Genetically altered mouse models have elucidated the *in vivo* role of PKGI in the cardiovascular system. Mice with whole-body PKGI deletion develop impaired vascular relaxation to acetylcholine or the cGMP analog 8Br-cGMP (11), and mice harboring discrete mutations in the PKGIα leucine/isoleucine zipper domain also develop hypertension, abnormal vascular relaxation, and impaired VSCM structure and function (12). These genetic models, therefore, demonstrate an unequivocal role for PKGI in the maintenance of cardiovascular homeostasis *in vivo*.

PKGI regulates VSMC function through a variety of mechanisms. PKGI inhibits VSMC contractility by regulating signaling pathways that control myosin phosphorylation (reviewed in Ref. 8). Additionally, PKGI signaling affects the VSMC phenotype via regulation of gene transcription (reviewed in Ref. 13). The two PKGI isoforms interact with and phosphorylate different VSMC targets and, thus, regulate VSMCs via isoform-specific mechanisms (14). PKGI substrates can regulate VSMC contractility both by modulating the level of intracellular calcium and by altering the sensitivity of the contractile apparatus to calcium (8). PKGIα binds to and phosphorylates the myosin binding subunit of myosin phosphatase and the regulator of G protein signaling 2 (RGS2) (15–17), whereas PKGIβ interacts with the inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate (IRAG) (14, 15, 18–21). Additional PKGI substrates in VSMCs include the thromboxane receptor (22), the inositol 1,4,5-trisphosphate receptor (23), phospholamban (24), and L-type calcium channels (25). PKGI regulates gene transcription by both direct and indirect mechanisms. PKGI can phosphorylate and, thereby, directly increase the transcriptional activity of the transcription factors cAMP response element-binding protein, ATF-1, and TFII-I (reviewed in Ref. 13). In addition, PKGIα directly binds RhoA (26), and PKGI-mediated phosphorylation inhibits RhoA and regulates gene transcription by inhibiting RhoA-mediated activation of the serum response factor (27).

Given these significant effects of PKGI in VSMCs, the identification of additional PKGI VSMC substrates may provide important insight into its role in regulating vascular function.

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[5] The abbreviations used are: VSMC, vascular smooth muscle cell; TXR-S, thromboxane receptor; IPTG, isopropyl 1-thio-β-D-galactopyranoside; pfu, plaque-forming units; SNOC, *S*-nitrosocysteine; MBS, myosin binding subunit; ANP, atrial natriuretic peptide.

Therefore, we employed a phosphorylation screen to isolate and identify physiological PKGI substrates. Fukunaga and Hunter (28) developed a novel expression screening method for identification of kinase substrates by screening a λ phage cDNA expression library for *in situ* solid-phase phosphorylation by purified protein kinase and [γ-^{32}P]ATP. They successfully identified a novel protein kinase, MAP kinase signal-integrating kinase (MNK1), as an ERK1 substrate.

In this report, we employed a similar strategy to screen for PKGI VSMC substrates. We describe the construction and screening of a human coronary artery smooth muscle cell library for phosphorylation by PKGI and our identification and characterization of steroid-sensitive gene 1 (SSG1) as a new PKGI substrate.

EXPERIMENTAL PROCEDURES

Preparation of a λGEX5 Coronary Artery Smooth Muscle Cell cDNA Library—Low-passage (passage 1–4) human coronary artery smooth muscle cells were lysed in an ice-cold denaturing solution (26 mM sodium citrate (pH 6.8), 0.5% *N*-laurylsarcosine, 0.125 M β-mercaptoethanol, and 4 M guanidine thiocyanate) using a total RNA isolation system (Promega). The lysate was mixed with sodium acetate and extracted with phenol:chloroform. Total RNA was obtained by isopropanol precipitation and washing with 70% ethanol. Approximately 1.9 mg of total RNA was obtained from 1.5×10^8 human coronary artery smooth muscle cells. Poly(A) mRNA was purified by hybridization to a biotinylated oligo(dT) primer. The mRNA was captured and washed at high stringency using streptavidin coupled to paramagnetic particles (Polytract mRNA isolation system, Promega). Approximately 16 μg of mRNA was isolated. cDNA was synthesized from poly(A) RNA using a cDNA synthesis kit (Promega). Adaptors consisting of 5′ phosphorylated oligonucleotides, pCCAGCACCTGCA and pAGGTGCTGG, were ligated to the cDNA. SfiI-digested λGEX5 arms were ligated to the cDNA and packaged into bacteriophage λ particles using an *in vitro* packaging reaction (Stratagene Gigapack Gold). The cDNA library contained ~960,000 independent clones. This library was amplified once by growth in *Escherichia coli* BB4 cells on agar plates prior to screening.

Construction of Positive Control Phage and Optimization of Screening Conditions—DNA fragments encoding the thromboxane receptor (TXR-S) myosin binding subunit (MBSC) PKG1α substrate sequences were amplified by PCR, digested with SfiI, ligated into λGEX5, and packaged into bacteriophage λ particles using an *in vitro* packaging reaction (Stratagene Gigapack Gold). λGEX5 was used as a negative control. λGEX5-M (myosin binding subunit) and λGEX5-T (TXR-S) were used as positive controls.

λGEX5, λGEX5-M, and λGEX5-T were plated with the *E. coli* BB4 strain at a density of 250 plaques/100-mm agar plate. After incubation at 42 °C for 3.5 h, the plates were overlaid with nitrocellulose membrane filters that were presoaked with 10 mM IPTG. After incubating for an additional 6 h at 37 °C, the plates were cooled to room temperature. The filters were marked with waterproof ink, peeled off the plates, and immersed in blocking solution (3% BSA, 1% Triton X-100, 100 mM NaCl, 20 mM Tris-HCl (pH 8.0)) for 1 h at room temperature or overnight at 4 °C. All filters were washed three times with Triton wash buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 1 mM DTT, and 0.2 mM PMSF) and once with PKG reaction buffer (50 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$). The filters were then incubated for 1 h with PKG buffer containing 0.1 mM ATP to mask proteins that autophosphorylate. After washing for 10 min in PKG reaction buffer containing 0.1 mM cGMP but without ATP, the filters were cut into small pieces (1.5 × 1.8 cm), each piece containing 5–25 plaques. The small filters were grouped into four. Each group included one negative (λGEX5) and two positive (one λGEX5-M and one λGEX5-T) controls. The four groups of filters were incubated for 1 h with PKG buffer containing 0.1 mM cGMP, 10μCi/ml [γ-^{32}P]ATP and different concentrations of purified PKG enzyme (1 μg/ml, 2 μg/ml, 4 μg/ml, and 6 μg/ml). The filters were then washed three times for 10 min with Triton wash buffer. A final wash in the absence of Triton was performed prior to phosphorimager analysis of the filters. The GST fusion protein expression by the phages was tested by Western blotting with anti-GST antibody (Amersham Biosciences Pharmacia Biotech) and anti-goat IgG antibody (Sigma).

Screening of a cDNA Library by Solid-phase Phosphorylation—The human coronary artery smooth muscle cell cDNA library was plated with the *E. coli* BB4 strain at a density of 1.9×10^4 plaques/150-mm agar plate. The plates were incubated and blocked as above. The plates were then overlaid with a second set of IPTG-soaked membranes and incubated overnight at 37 °C to generate duplicate lifts. All filters were washed three times with Triton wash buffer, and once with PKG reaction buffer. The filters were then incubated for 1 h with PKG buffer containing 0.1 mM ATP. Following washing for 10 min in PKG reaction buffer containing 50 μM cGMP but without ATP, the filters were incubated for 1 h with PKG reaction buffer containing 0.1 mM cGMP, 10μCi/ml [γ-^{32}P]ATP and 6 μg/ml purified G kinase enzyme. The filters were then washed three times for 10 min with Triton wash buffer. A final wash in the absence of Triton was performed prior to phosphorimager analysis of the filters. Positive clones were identified, and the plaques were purified by plating at a lower density (200–800 pfu/100-mm dish). Secondary screening of these clones was undertaken to discriminate between false positive signals and true positive plaques. These cDNAs were rescued as expression plasmids, and their cognate proteins were expressed in *E. coli*, purified by glutathione-agarose, and tested for phosphorylation by PKGI. Although all of the recombinant products were phosphorylated by PKGI *in vitro*, six of the clones produced GST fusion proteins with a very short tail that were thought to be artificial products derived from out-of-frame ligations.

Conversion of Phage Clones into Plasmids—For further characterization, positive clones were converted into plasmids containing the cDNA inserts using a rapid plate lysate method. Briefly, *E. coli* BB4 infected with phage was treated with λ diluent (10 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$) containing 2 μg/ml DNase I and 20 μg/ml RNase A for 2 h. After extraction with chloroform to remove bacterial debris, phage particles were isolated by precipitation with 20% polyethylene glycol 8000 and 2 M NaCl. Phage DNA was obtained by dissolving the

phage pellet in extraction buffer (10 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 10 mM EDTA, 0.1% SDS) for 10 min at 68 °C, followed by phenol:chloroform extraction and ethanol precipitation. Phage DNA was digested with Not1 for 3 h at 37 °C and then heated for 20 min at 70 °C. Plasmid cDNA (pGEX-PUC-3T) was recovered by self-ligation with T4 DNA ligase followed by transformation of *E. coli* XL1-blue. Overnight cultures of the transformed bacteria were used for recombinant plasmid DNA preparation and GST fusion protein preparation after IPTG induction.

Identification of cDNA Clones Encoding Substrate Candidates—Purification and analysis of GST fusion proteins encoded by candidate clones were performed as described previously (22). Briefly, cultures of transformed bacteria were used for plasmid DNA preparation and sequencing as well as purification of GST fusion proteins with glutathione-agarose (Sigma) after IPTG induction, followed by a Basic Local Alignment Search Tool (BLAST) search. Potential PKG phosphorylation sites were detected using the Simple Modular Architecture Research Tool. The *in vitro* phosphorylation reaction with purified GST fusion proteins was performed in 50 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM cGMP, and 0.48 μg/ml purified PKG1 and was initiated by the addition of 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol, Dupont/NEN) for 10 min at room temperature (total reaction volume, 50 μl). The reaction was stopped by the addition of protein sample buffer and heating for 3 min at 100 °C. The proteins were separated by polyacrylamide gel electrophoresis, and the phosphorylated proteins were visualized using a Molecular Dynamics PhosphorImager system.

Library Screening Hybridization—The human coronary artery smooth muscle cell cDNA library was plated on *E. coli* XL1-blue strain at a density of 5×10^4 pfu/150-mm agar plate. After incubation at 37 °C for 9 h, the plates were chilled for 2 h at 4 °C, after which nitrocellulose filters were placed for 2 min and a second filter for 4 min. The filters were denatured in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 2 min, neutralized in neutralization solution (1.5 M NaCl, 0.5 M Tris-Cl (pH 8.0)) for 5 min, and rinsed in wash solution (0.2 M Tris-Cl (pH 7.5), 2 × SSC) for 30 s. The filters were blot-dried on Whatman 3MM paper, and the DNA was cross-linked to the filters using a UV Stratalinker 2400 (Stratagene). The filters were prehybridized in prewarmed (55 °C) hybridization solution (5 × SSC, 5 × Denhardt's solution, 1% SDS, 100 μg/ml denatured salmon sperm DNA) at 55 °C for 3 h and then hybridized in prewarmed (55 °C) hybridization solution containing 1–15 ng/ml of denatured ^{32}P-labeled probe at 55 °C overnight. The filters bearing the hybridized DNA were washed in prewarmed (45 °C) wash buffer I (2 × SSC, 0.5% SDS) at 45 °C for 10 min and then washed in prewarmed (45 °C) wash buffer II (0.05% SSC, 0.1% SDS) for 2 × 10 min at 45 °C. After washing, each filter was exposed to x-ray film or a PhosphorImager screen overnight. Positive clones were identified and purified by plating at a lower density (50–100 pfu/100-mm dish). The phage DNA was prepared from the positive clones and digested with EcoR1. The insert DNA fragments were analyzed by DNA sequencing, subcloning, mapping, and expression.

Radioactive Probes—The ^{32}P-labeled probe was prepared by digesting plasmid DNA C3-3 with BamHI and NotI (BioLabs). The 323bp DNA fragment was purified by low melting point-agarose (Invitrogen) using the QIAquick gel extraction kit (Qiagen) and labeled with [α-^{32}P]dCTP using the Rediprime DNA labeling system (Amersham Biosciences Life Science).

Preparation of Phagemid DNA—Preparation of phagemid DNA was performed using the ExAssist kit (Stratagene). Briefly, 200 μl of XL1-blue MRF cells (A_{600} = 1.0), 250 μl of phage stock (> 1×10^5 phage particles), and ExAssist helper phage (> 1×10^6 pfu/ml) were mixed in a tube. After 15-min incubation at 37 °C, 3 ml of NZY medium (5 g/liter NaCl, 2 g/liter $MgSO_4$ $7H_2O$, 5 g/liter yeast extract, 10 g/liter casein hydrolysate, 15 g/liter agar) was added to the mixture and incubated for 2.5–3 h at 37 °C with shaking. The tube was heated at 65–70 °C for 20 min. To plate the rescued phagemid, 200 μl of freshly grown XLOLR cells (A_{600} = 1.0) and 10–100 μl of phage stock were incubated at 37 °C for 15 min. 300 μl of NZY medium was added, incubated at 37 °C for 45 min, and then 200 μl of cells were plated on LB/kanamycin (50 μg/ml). The plate was incubated overnight at 37 °C. The colonies appearing on the plate contained the pBK-CMV double-stranded phagemid with the cloned DNA insert. The phagemid DNA was prepared from overnight cultures of a single colony grown in LB medium containing 50 μg/ml of kanamycin at 37 °C. The DNA was digested with EcoRI, sequenced, and followed by a BLAST search.

Primers for Sequencing H10-1 DNA—Nucleotides 1–810 of H10-1 DNA were 99% identical to 139046–139880 of a human BAC DNA library sequence (ACO48334) with the sequence of C3-3 located between 140011 and 140244. The human BAC gene sequence, including the C3-3 sequence, was used to make three primers to sequence H10-1. The primers were as follows: 3′BAC1 (139459), GCTCACAGTACACATC GTCC; 5′BAC2 (139316), CAGGGAAGAACAGAGTATGG; and 5′BAC5(139854), GAAGAAAGAGGACCCAAGG.

Site-directed Mutagenesis—Mutagenesis of the proximal serine (Ser-49) and the distal serine (Ser-74) of plasmid C3-3 was performed using the QuikChange mutagenesis kit (Stratagene) according to the instructions of the manufacturer. Two single-stranded 28-mer mutagenic primers corresponding to Ser-49 and two single-stranded 27-mer mutagenic primers corresponding to Ser-74 were used for site-directed mutagenesis to change serines to alanines. The following oligonucleotides were used: Ser-49, 5′-GCC AGG AGA CCC GCG GTT TCA GAG AAT C and Ser-74 5′-ACC AGG AGG CCC GCC AAG GCC ACC AGC. PCR products digested with DpnI (BioLabs) to remove the parental dsDNA, followed by transformation into *E. coli* XL1-blue. The plasmid DNA was purified from transformants by the Rapid Plasmid purification system (Invitrogen) and sequenced. The double mutant construct S49/74A was generated identically by using the S49A primer and S74A as a template. Following selection and screening, the mutant constructs were verified by DNA sequencing and *in vitro* phosphorylation of GST fusion proteins.

Cloning of Human SSG1—The human SSG1 (hSSG1) and truncated hSSG1 DNA fragments and the N-terminal (hSSG1N, 1–257) and C-terminal fragment (hSSG1C, 258–557), were amplified from the phagemid H10-1 DNA by PCR using pfu DNA polymerase (Stratagene). To facilitate the sub-

sequent cloning steps, a BamH1 restriction site was introduced in the 5′ primers, and a Sal1 site was introduced in the 3′ primers. The PCR products were digested with BamH1-Sal1 and inserted into the plasmid DNA pET-28a(+), and pCMV-Tag2B. *E. coli* XL10-gold-competent cells were transformed with the recombinant DNA. Isolated clones were identified by restriction digestion and confirmed by sequencing. For protein expression of hSSG1, hSSG1N, and hSSG1C, the recombinant DNA was transformed into host strain BL21(DE3), grown in LB containing 50 μg/ml kanamycin, and then protein expression was induced with 0.2 mM IPTG for 2.5 h at 37 °C. Protein purification was achieved with the protocol for batch purification under denaturing conditions using nickel-nitrilotriacetic acid HisBind resin (Novagen). Immunoblotting was performed with anti-C3-3 and anti-His_6 (Roche) antibodies. For protein expression of cloned hSSG1, hSSG1N, and hSSG1C DNA in HEK293 cells, cDNAs in vector pCMV-Tag2B were transiently transfected using PolyFect transfection reagent (Qiagen) according to the instructions of the manufacturer. After a 48-h incubation, cells were harvested using TLB buffer (20 mM Tris.Cl, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 25 mM β-glycerol phosphate (pH 7.4)) with a protease inhibitor mixture (SetIII, Calbiochem), and the expressed hSSG1 protein was immunoprecipitated using an anti-FLAG M2 affinity gel (Sigma). Immunoblotting was performed using either anti-FLAG M2 monoclonal antibody (Sigma) or anti-C3-3 antibody.

In Vitro Cointeraction Studies—For detection of the direct interaction between PKGI and SSG1, 10 μl of ^{35}S-labeled hSSG1 protein was mixed with 1.2 μg of purified PKGI and incubated for 2 h at 4 °C. 0.1 mM cGMP was added to some of the reactions, which were then incubated for 30 min at 37 °C and followed by immunoprecipitation with antibody to PKGI or nonimmune IgG.

Northern Blot Analysis—The tissue distribution of hSSG1 message was assessed by Northern blot analysis. Multiple-tissue Northern (MTN™) blots, each containing ~2 μg of poly (A)+ RNA/lane, were purchased from Clontech (human 12-lane tissues, catalog no. 7780-1, and human cardiovascular tissues, catalog no. 7791-1). Hybridization was performed under stringent conditions in ExpressHyb according to the instructions of the manufacturer. The blots were probed with a ^{32}P-labeled, 323-bp BamH1/Not1 fragment of plasmid DNA C3-3. The hybridized blots were exposed to x-ray film (Kodak) between intensifying screens at −80 °C for 4 h or overnight.

Immunostaining—Co396 and Ao184 cells, grown on coverslips in 12-well plates, were fixed with 3.7% formaldehyde at room temperature for 15 min, rinsed with PBS, and permeabilized with PBS containing 0.3% Triton X-100 and 10% donkey serum for 15 min. After blocking with 10% serum for 1 h, the cells were incubated with a normal rabbit IgG or anti-C3-3 IgG preabsorbed with GST for 1 h at room temperature. After rinsing, a Cy3-linked anti-rabbit secondary antibody (1:1000) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added for 1 h. The cells were rinsed and incubated with DAPI/PBS for 15 min. After additional washing steps, the coverslips were mounted with anti-fade medium (Molecular Probes, Eugene, OR). Single and merged images were prepared using a Nikon OptiPhot-2 microscope with fluorescent attachment and a charge-coupled device camera.

Subcellular Fractionation of SSG1—Co396 cells were grown to 80% confluence in 100-mm dishes, rinsed, and serum-deprived overnight. The cells were treated with the indicated concentration of agonist, rinsed, and harvested into 500 μl TLB (10 mM Hepes, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM EDTA and protease inhibitor mixture (Set III, Calbiochem, La Jolla, CA)). The cell suspension was homogenized 50 times on ice. The cell lysate was centrifuged at 7500 × *g* for 5 min. The supernatant was removed and recentrifuged in a TLA120.2 rotor at 25,000 rpm (27,181 × *g*) for 30 min. (Beckman Optima™ TLX ultracentrifuge). The supernatant was removed and designated as the cytosolic fraction, and the pellet was resuspended in 40 μl of PBS and designated as the crude plasma membrane fraction. The pellet from the first centrifuge was resuspended in 1 ml of TSE (10 mM Tris.Cl (pH 7.5), 300 mM sucrose, 1 mM EDTA, 0.1% NP_{40}, and protease inhibitor mixture (SetIII, Calbiochem)), homogenized 30 times on ice, and centrifuged at 5000 rpm (1087 × *g*) for 5 min. The pellet was washed twice with TSE buffer, resuspended in 40 μl of TSE buffer, and designated as the nuclear fraction. All final fractions were suspended in an equal volume of protein loading buffer (2×), heated to 100 °C for 5 min, and analyzed by Western blot analysis with anti-C3-3 IgG antibody.

^{32}P Labeling of SSG1 and Immunoprecipitation—Ao184 (human aortic smooth muscle cells) were washed with phosphate-free DMEM and incubated overnight. Phosphate-free DMEM with 0.625 mCi [^{32}P]orthophosphate was then added for 4 h. After 20 μM *S*-nitrosocysteine (SNOC) treatment for 10 min, the cells were washed with ice-cold PBS and lysed in 0.5 ml ice-cold lysis buffer containing 40 mM Tris.Cl (pH 7.5), 274 mM NaCl, 4 mM EDTA, 2% Triton X-100, 20% glycerol, 50 mM β-glycerol phosphate, 1 mM PMSF, and protease inhibitor mixture (SetIII, Calbiochem)). The lysates were centrifuged at 16,000 × *g* for 15 min at 4 °C after 1-h incubation on ice, and the supernatant of ^{32}P-labeled solubilized proteins was used for immunoprecipitation. For immunoprecipitation of ^{32}P-labeled SSG1, 3 μl of anti-C3-3 rabbit antiserum was added to each sample and incubated overnight at 4 °C. 30 μl of protein A was added for 1.5 h. The pellet was washed three times with wash buffer (50 mM Tris.Cl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, and 0.1% SDS). Isolated proteins were analyzed by 7.5% SDS-PAGE. The gel was stained with Coomassie Blue, destained with 10% acetic acid, and dried. The dried gel was exposed to Kodak film at −80 °C for 6 h. ^{32}P-Labeled SSG1 was quantitated in duplicate by autoradiography.

For the ANP phosphorylation studies, Ao184 cells were treated for 10 min with atrial natriuretic peptide (Sigma) at 0.1 ng/ml, 1 ng/ml, and 10 ng/ml. Cells were lysed, and SSG1 IP was performed exactly as described above. Western blotting was performed with anti-phosphoserine antibody (Sigma, 1:800 dilution), and blots were stripped and reprobed with SSG1 antibody.

Preparation and Preliminary Identification of Phage Clone DNA—Preparation of the clone phage DNA was as described above for the conversion of phage clones into plasmids, except

that the bacterial strain XL1-blue was used instead of *E. coli* BB4. The phage DNA was digested with EcoR1 and run on a 0.8% agarose gel followed by Southern hybridization. The probe used was the C3-3 clone as described above.

Preparation and Analysis of Antibodies—GST-C3-3 fusion protein was purified with glutathione-agarose (Sigma). Proteins were separated on a 10% SDS-polyacrylamide gel, labeled by Coomassie stain, and the band corresponding to the GST-C3-3 fusion protein was excised. The purified peptide was sent to Alpha Diagnostic International, Inc. (San Antonio, TX), where it was used to immunize rabbits. The animals were bled at week 0 for preimmune and at weeks 7, 9, and 11 for analysis after the first injection. The analysis of the antiserum was performed by Western blot analysis.

In Vitro Transcription-Translation and Binding of PKGI and SSG1—In vitro transcription-translation was performed using the TNT kit (Promega) as directed by the manufacturer using 1 μg of the expression phagemid DNA (H1-1, H8-1, H10-1, and H12-3) and cloned hSSG1 cDNA (pcSSG1(1), pcSSG1(2), and pcSSG1(3)). [^{35}S]methionine (Amersham Biosciences Pharmacia, catalog no. AG1049) was incorporated into nascent protein. A 5-μl aliquot was removed and mixed with 20 μl of SDS sample buffer. After heating at 100 °C for 2 min, 5–10 μl of the denatured sample was resolved by 12% SDS-PAGE and subjected to autoradiography.

Statistical Analyses—Data are presented as mean ± S.E. A two-tailed Student's *t* test was used. Values of $p < 0.05$ were considered statistically significant. All experiments were repeated at least three times.

RESULTS

Phosphorylation Screen of a Human Smooth Muscle Expression Library Identifies SSG1 as a PKGI Substrate—Others have previously developed and performed a phosphorylation screen of a cDNA expression library to identify and isolate a novel MAP kinase substrate (28). To validate this approach for the identification of PKGI substrates, we first tested two known PKGIα substrates, the thromboxane receptor (22) and the myosin binding subunit (MBS) of myosin phosphatase (15). Purified PKG phosphorylated each of the two control substrates expressed from a λGEX5 vector at a resolution of 1:250 when mixed with random λcDNAs (Fig. 1).

To identify novel PKG1 substrates, we constructed an HCASMC cDNA library containing 5×10^5 independent clones and screened for phosphorylation with purified PKGI (Fig. 2). Among 15 isolated positive clones, eight produced phosphorylated GST fusion proteins with an insert size between 5 and 13 kDa (Fig. 3*A*). The sequence of the inserts was determined and compared for identity/homology by searching with BLAST on the National Center for Biotechnology Information file server. Although the amino acid sequences of all the proteins contained possible PKGI phosphorylation motifs, RR*X*S or RK*X*S (29), none of them corresponded to a known PKGI substrate.

One of the identified polypeptides, C3-3, was chosen for further analysis on the basis of its high level of phosphorylation (Fig. 3*A*). C3-3 is 123 amino acids in length and contains nine serines, 15 threonines, and one tyrosine (Fig. 3*B*). Two predicted PKG phosphorylation sites resided at residues 49 and 74. To identify the full-length cDNA that contains C3-3, we screened the human CASMC library using C3-3 DNA as a probe. Fifteen clones were identified from 5×10^5 plaques. Southern blot analysis of the clones using C3-3 DNA as a probe revealed an insert size ranging from 1.2–8.3 kb (Fig. 4*A*). The longest inserts (clones H1-1/H10-1, 3.8 kb) were subcloned into a phagemid vector and sequenced (Fig. 4*B*). A GenBank search revealed that nucleotides 44–1701 of H1-1/H10-1 matched nucleotides 138916–140573 of a human BAC DNA (ACO48334) 100% (Fig. 4*C*). The C3-3 fragment is located between 140011 and 140244 of the BAC DNA. The H1-1/

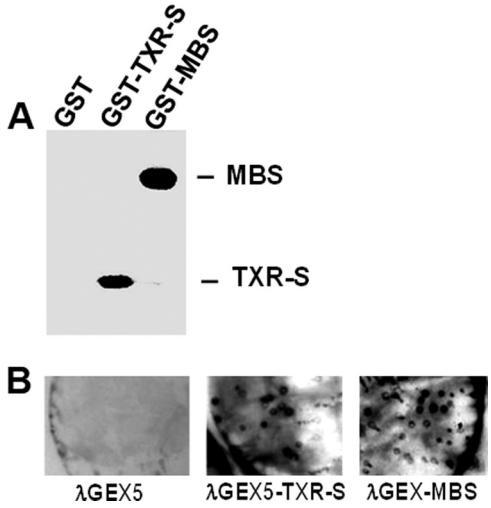

FIGURE 1. **Optimization of PKGI phosphorylation screen and confirmation of phosphorylation of known PKGI substrates.** *A*, GST, the GST-thromboxane receptor C-terminal tail domain (GST-TXR-S), and the GST-MBS C-terminal domain (GST-MBS) were phosphorylated by PKGI *in vitro* and subjected to SDS-PAGE and autoradiography. The positions of GST-TXR-S and GST-MBS are indicated by *dashes*. *B*, phosphorylation by PKGI of TXR-S and MBS in λGEX5. TXR-S and MBS were packaged into bacteriophage λ particles, plated with the *E. coli* BB4 strain, and overlaid with nitrocellulose membrane filters that were subsequently phosphorylated with PKGI as described under "Experimental Procedures." The phosphorimager analysis of the filters is shown. Results are representative of three separate experiments.

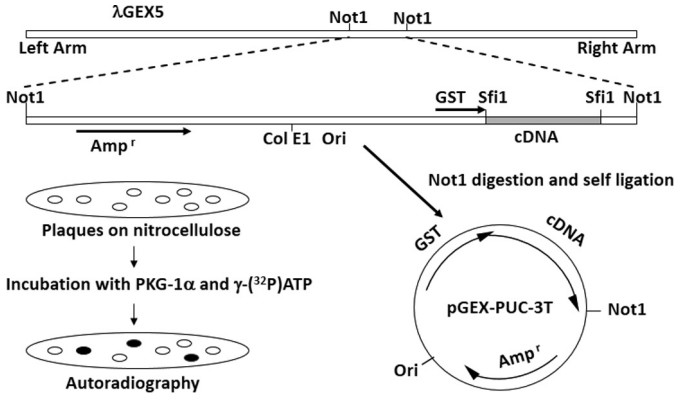

FIGURE 2. **PKGI phosphorylation screen.** The human coronary artery smooth muscle cell cDNA library was ligated into the Sfi1 sites downstream of a GST sequence and an ampicillin resistance (*Amp^r*) sequence. This bacterial expression cassette was ligated into the Not1 sites of λ phage DNA. Phage plaques were transferred to nitrocellulose, incubated with purified PKGI, and subjected to autoradiography to identify potential substrates (as shown on the *left*). For putative clones, the bacterial expression cassette was excised with Not1, circularized by self-ligation, and then the GST fusion proteins were expressed and purified for subsequent phosphorylation reactions (as shown on the *right*). *ColE1*, colicin E1 carrying plasmid; *Ori*, origin of replication.

H10-1 sequence was also 78% similar to rat steroid sensitive gene 1 (R-SSG1) (30).

Fig. 5 shows the nucleotide sequence and the predicted amino acid sequence of human SSG1 (hSSG1) in which the C3-3 fragment is *underlined*. hSSG1 is 1671 base pairs in length, encoding a protein of 557 amino acids, with a predicted molecular weight of 61.2 kDa. Other notable domains of hSSG1 include a putative cleavable signal peptide (amino acids 1–21, *red*); several potential nuclear localization signals at amino acids 73 (PLQRRRS), 492 (PPKKKAQ), and 493 (PKKKAQD); and bipartite nuclear localization signals at amino acids 419 (RKDQHRERPQTTRRPSK) and 538 (KKHEKLEKPEKEKKNS). A threonine-rich region was found at amino acids 347–404, and a lysine-rich region was found at amino acids 486–551.

Five predicted PKG1 recognition sequences were noted, and two of them (serine 409 and serine 434) are located in the C3-3 region. The carboxyl terminus of hSSG1 (amino acids 404–529) shares 63% amino acid sequence identity with the amino terminus of R-SSG1 (amino acids 3–127) (30). The amino terminus of hSSG1 (amino acids 1–537) shares 99% amino acid sequence identity with the amino terminus of human protein known as up-regulated in bombesin-like receptor 3-deficient mice (URB) a protein that was originally identified in human bone marrow stromal cells with an open reading frame of 950 amino acid residues (31).

Human SSG1 Protein Associates with and Is Phosphorylated by PKGI—The predicted size of hSSG1 is 61.2 kDa. Using the phagemid DNA H1-1and H10-1 as templates for *in vitro* transcription and translation revealed a protein of 62.0 kDa, in close agreement with the predicted size (Fig. 6*A*). The size of hSSG1 was confirmed by using the cloned hSSG1 DNA pcSSG1(1), pcSSG1(2), and pcSSG1(3) as templates (Fig. 6*B*).

We next tested for direct cointeraction of PKGI and SSG1. The ^{35}S-labeled hSSG1 protein was incubated with purified PKGI and precipitated with anti-PKGI antibody. hSSG1 coprecipitated with PKGI protein *in vitro* (Fig. 7), suggesting direct binding of PKGI and hSSG1. We also examined whether cGMP activation of PKGIα regulated its binding to SSG1. Activation of PKGI with cGMP induced a decrease in PKGI-SSG1 interaction (Fig. 7).

To test whether PKGI directly phosphorylates SSG1, we immunoprecipitated SSG1 from Ao184 cell lysates using the C3-3 antibody and then incubated SSG1 with increasing concentrations of purified PKGIα with or without cGMP. Increasing concentrations of PKGIα increased the incorporation of ^{32}P into the SSG1 band, and this phosphorylation increased further in the presence of cGMP (Fig. 8*A*). We empirically determined

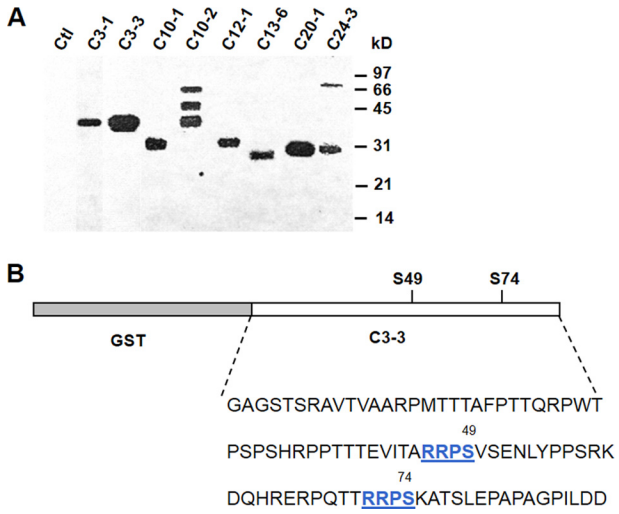

FIGURE 3. **Phosphorylation of putative PKGI substrates expressed as GST fusion proteins in bacteria.** *A*, autoradiogram of clones isolated from the PKGI phosphorylation screen. The phage clones were purified as described under "Experimental Procedures" and expressed as GST fusion protein in bacteria, followed by purification and phosphorylation by PKGI. The phosphorylated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to autoradiography. *Ctl*, GST alone. Clone C3-3 was selected for further analysis. *B*, schematic showing clone GST-C3-3 with the predicted PKGI phosphorylation sites at serine 49 and 74 as well as the amino acid sequence. The predicted PKGI phosphorylation sites are *underlined* and *blue*. Results are representative of three separate experiments.

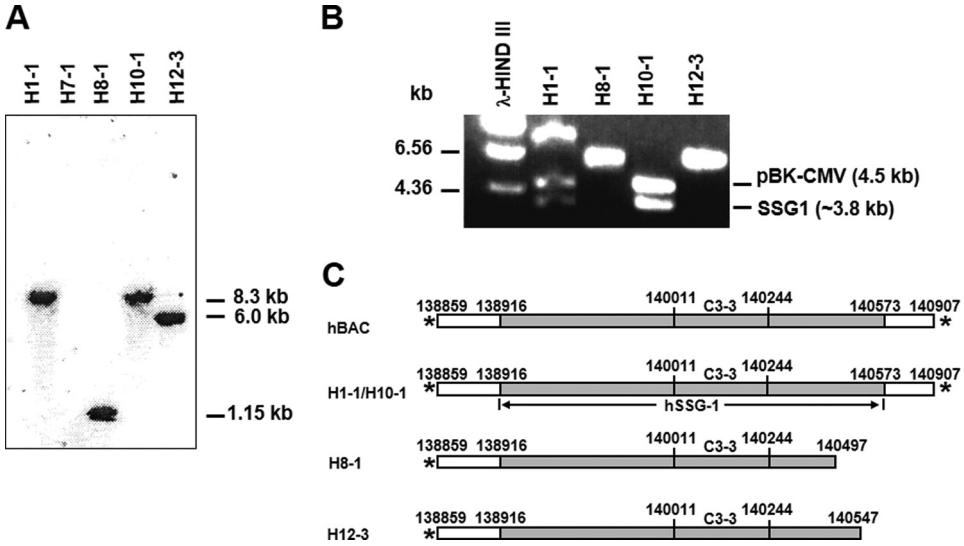

FIGURE 4. **Analysis of clones from the human aorta cDNA library using C3-3 as a probe.** *A*, Southern blot analysis. The digested phage DNA prepared from the positive clones was hybridized with a ^{32}P-labeled C3-3 DNA probe as described under "Experimental Procedures." *B*, phagemid DNA was prepared from the positive phage clones, digested with EcoR1, and separated by a 1% agarose gel. The *arrows* indicate the 3.8-kb full-length hSSG1, as confirmed by DNA sequencing, and the vector DNA. Results are representative of three separate experiments. *C*, schematic representation of the H1-1/H10-1, H8-1, and H12-3 sequences and their positions within the hBAC clone. The hBAC sequence was from the Roswell Park Cancer Institute Human BAC library.

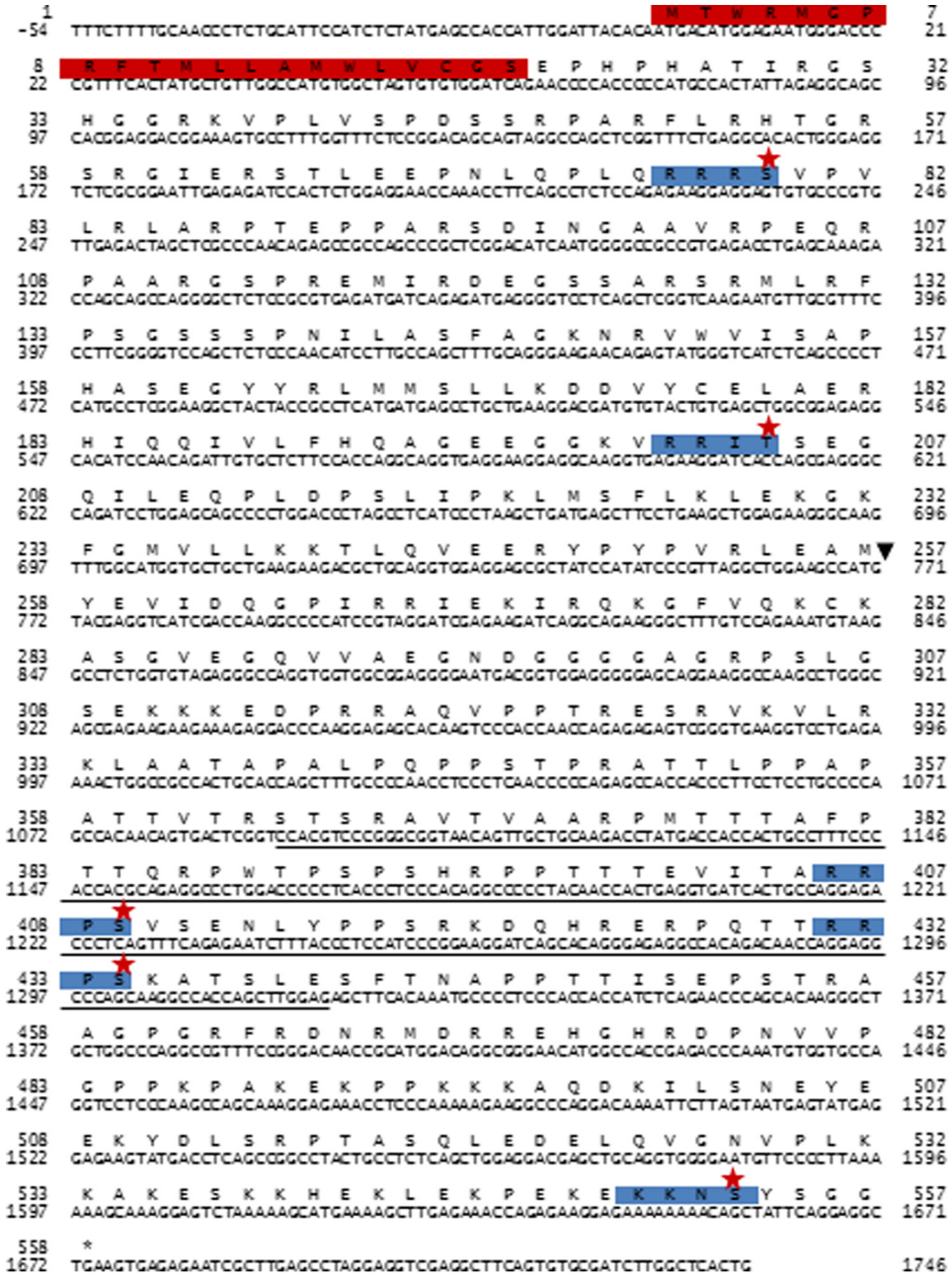

FIGURE 5. **Nucleotide sequence and predicted amino acid sequence of human gene SSG1.** The *underlined* sequence specifies the C3-3 portion of the protein. The putative cleavable signal peptide is shaded in *red*. The predicted PKGI phosphorylation sites are marked by *red stars*. The truncating site of the sequence is marked by an *arrowhead*. The stop codon is marked by an *asterisk*.

the stoichiometry of this process to be 2 mol of phosphate per 1 mol of SSG1 for unstimulated PKGIα and 3 mol of phosphate per 1 mol of SSG1 for cGMP-stimulated PKGIα (not shown).

We therefore hypothesized that there are at least two basal PKGI phosphorylation sites on SSG1. To test this, we mutated the predicted PKGI phosphorylation sites Ser-49 and Ser-74 of the polypeptide C3-3 to alanines using site-specific mutagenesis. Although wild-type C3-3 was phosphorylated by PKGI, phosphorylation of the S49A and S74A mutants by PKGI was decreased, and detection of phosphorylation was abolished completely in the double-mutated S49/74A protein (Fig. 8, *B–D*). These data support that both Ser-49 and Ser-74 in C3-3 are phosphorylated normally by basal PKGI activity.

To examine the relevance of the PKGI-SSG1 interaction in intact VSMCs, we tested, in Ao184 cells, whether PKGI activation with the physiologic guanylate cyclase activator atrial natriuretic peptide (ANP) induced SSG1 phosphorylation. ANP increased SSG1 phosphorylation in a dose-dependent manner, as determined by anti-phosphoserine Western blot analysis from immunoprecipitated SSG1 (Fig. 9*A*). Additionally, the NO donor SNOC also increased SSG1 phosphorylation, as determined by ^{32}P autoradiography (Fig. 9*B*) in the same experimental system.

hSSG1 Is Widely Distributed in Cardiovascular Tissue and Cells—Because PKGI is highly expressed in cardiovascular tissue and smooth muscle cells (8), we examined whether SSG1 expresses in a similar pattern of tissues. We therefore per-

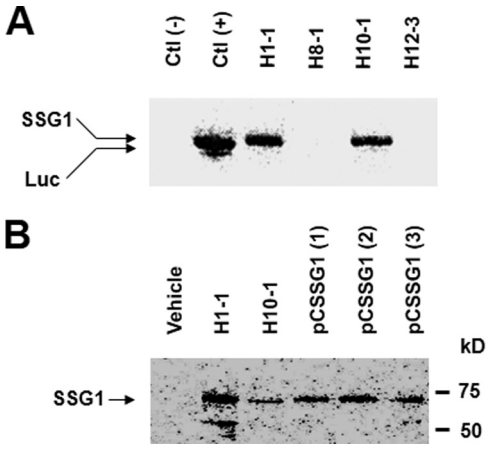

FIGURE 6. ***In vitro* transcription/translation of the phagemid and cloned hSSG1 cDNA.** *A*, *Ctl(−)*, no added DNA negative control; *Ctl(+)*, translated products of luciferase (*Luc*) cDNA used as a positive control. The H1-1, H8-1, H10-1, and H12-3 lanes show the translated products of the phagemid cDNA clones. *B*, the vehicle lane shows the translated product of vector DNA (pCMV-Tag2B). The H1-1 and H10-1 show the phagemid cDNA-translated products. The pCSSG1(1), pCSSG1(2), and pCSSG1(3) lanes show individually cloned hSSG1 cDNA-translated products. The molecular weight of luciferase is 61 kDa, and hSSG1 is 62.1 kDa.

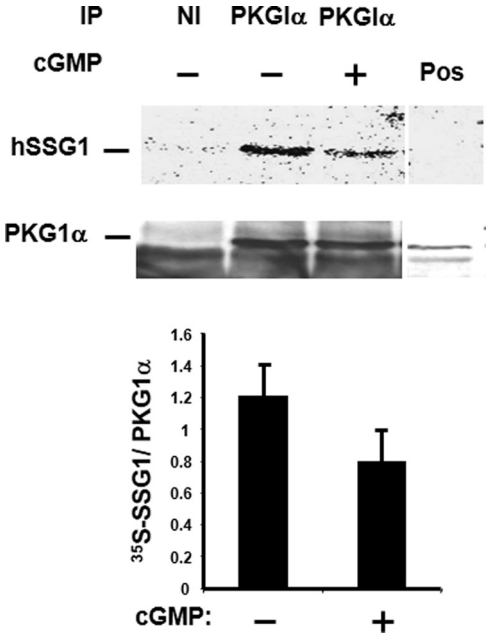

FIGURE 7. **Direct interaction of hSSG1 protein with PKGI *in vitro*.** Purified PKGI was mixed with ^{35}S-labeled hSSG1 and immunoprecipitated (*IP*) with anti-PKGI antibodies. *Top panel*, autoradiograph of ^{35}S-labeled hSSG1 bound to PKGI. *Center panel*, immunoblot analysis of PKG1 bound to the anti-PKG1 antibody. *Bottom panel*, densitometric analysis of ^{35}S-SSG1 bound to PKG1. *, $p < 0.05$. $n = 3$ experiments. *NI*, Non-immune antibody; *Pos*, purified PKGI-positive control.

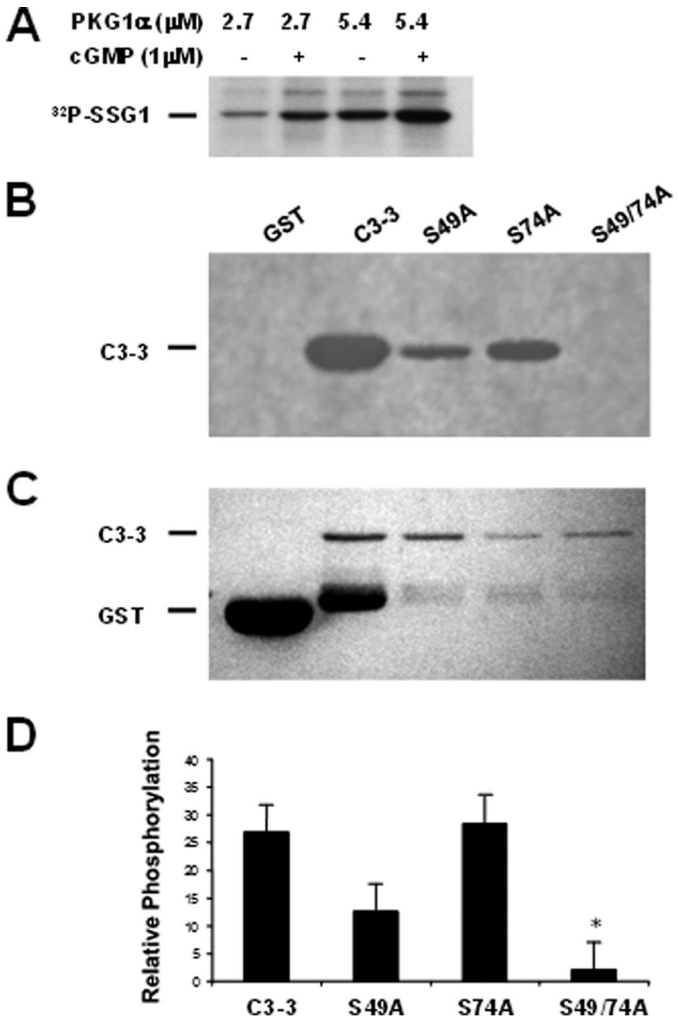

FIGURE 8. **Phosphorylation of SSG1 by PKGI *in vitro* (*A*) cGMP and PKGIα-dependent phosphorylation of SSG1 *in vitro*.** SSG1 was immunoprecipitated from Ao184 cell lysates, followed by incubation at 30 °C for 20 min with 2.7 or 5.4 μM purified PKGIα and 1 μM cGMP or vehicle. *B*, the bacterially expressed and purified GST, GST-C3-3, and GST-C3-3 mutants S49A, S74A, and S49/74A were phosphorylated by purified PKGI and then subjected to SDS-PAGE and autoradiography. The *arrow* indicates the position of phosphorylated GST-C3-3. *C*, Coomassie-stained SDS-PAGE of GST and GST fusion proteins from *B*. The *arrows* indicate the positions of GST and GST-C3-3. *D*, quantitation of phospho/total SSG1 C3-3 from *B* and *C*. *, $p < 0.05$.

formed a Northern blot analysis for hSSG1 in human tissues. We observed the highest hSSG1 expression in the heart, followed by skeletal muscle and colon (Fig. 10*A*). Lower expression levels were observed in the thymus, spleen, kidney, liver, small intestine, placenta, and lung. No expression was detected in the brain or peripheral blood leukocytes. The SSG1 transcripts are 3.8 and 3.4 kb in size, with an additional transcript of 1.8 kb detected in skeletal muscle tissue. We next examined the relative expression of SSG1 mRNA within specific human cardiovascular tissues and observed the highest SSG1 expression in the aorta, followed by the atria, right ventricle, left ventricle, and apex of the heart (Fig. 10*B*).

We also confirmed the protein expression of SSG1 in both vascular and non-vascular cell lines. The 62.0-kDa SSG1 protein was detected by immunoblot analysis in lysates from all 18 cell lines tested, including vascular smooth muscle cells derived from aorta, coronary artery, radial and mammary artery, and endothelial cells from aorta and umbilical vein (Fig. 10*C*). The 62-kDa protein band could not be detected when non-immune serum was used for the immunoblot analysis (data not shown). Similarly, preabsorption of the SSG1 antibody with SSG1 immunizing peptide blocked the detection of the 62-kDa band on immunoblot analysis (Fig. 11).

Subcellular Localization and Translocation of SSG1 by cGMP—PKGI regulates a number of its kinase targets, such as RhoA (26) and RGS2 (19), by altering their intracellular local-

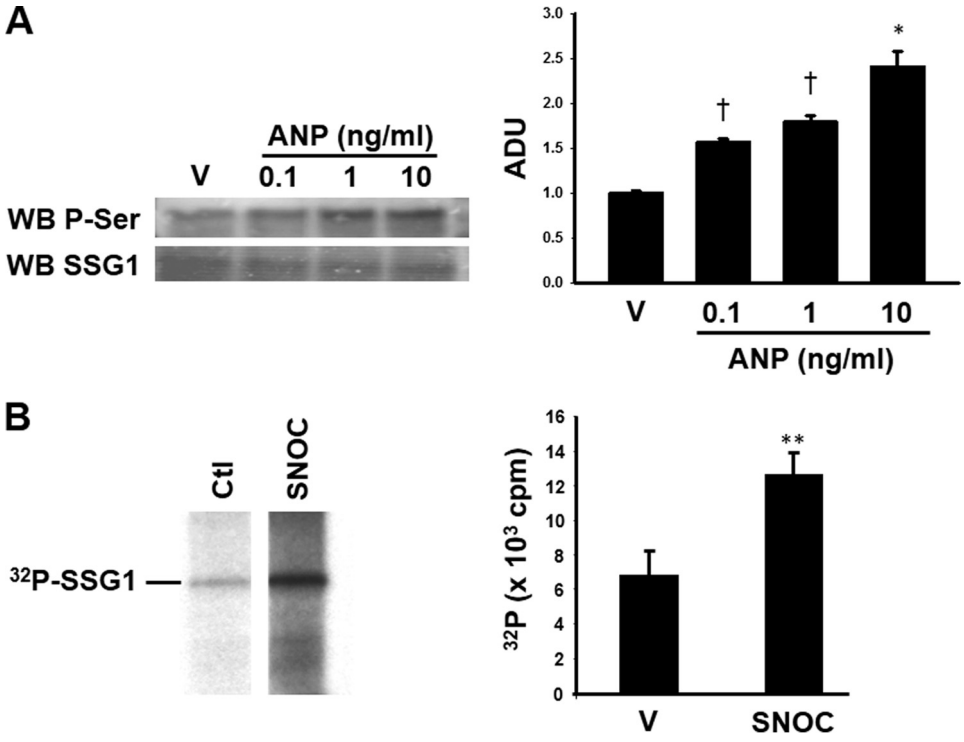

FIGURE 9. **Phosphorylation of SSG1 by PKGI-activating molecules in vascular smooth muscle cells.** *A*, Western blot analysis for phosphoserine or total SSG1 in SSG1 immunoprecipitates from Ao184 cells pretreated with ANP at 37 °C for 10 min. *, $p < 0.01$ *versus* all other groups; †, $p < 0.01$ *versus* vehicle. *B*, ^{32}P incorporation detected in SSG1 immunoprecipitate from Ao184 cells pretreated for 10 min with vehicle or with 20 μM of the NO donor SNOC. $n = 3$. **, $p = 0.05$. *V*, vehicle-treated.

ization. Therefore, we tested whether PKGI activation alters the subcellular localization of SSG1. We tested this using immunofluorescence microscopy and subcellular fractionation experiments. In unstimulated human coronary artery smooth muscle cells (Co396), we detected hSSG1 primarily in the nucleus and the cell membrane, as shown by anti-hSSG1 antibody labeling and immunofluorescence microscopy (Fig. 12*A*). Biochemical cell fractionation also revealed that ~90% of total hSSG1 is located in the nuclear fraction and ~10% is in the membrane fraction (Fig. 12*B*). We observed a similar distribution profile in other vascular smooth muscle cells (Ao184 human aortic smooth muscle cells, data not shown). Treatment of Co396 cells with increasing concentrations of the nonhydrolyzable, membrane-permeable cGMP analog 8Br-cGMP for 15 min significantly increased the portion of the hSSG1 in the cytosol (Fig. 13*A*). Subcellular fractionation studies confirmed that 8Br-cGMP treatment increased the cytosolic-to-membrane fraction of SSG1 (Fig. 13*B*).

DISCUSSION

This study sought to identify novel substrates of cGMP-dependent protein kinase in human vascular cells using an expression library kinase screen. Our screen identified a 62-kDa human protein cloned previously from rat uterus and known as SSG1. We report that hSSG1 directly interacts with PKGI and is specifically phosphorylated by PKGI at serines 409 and 434. cGMP activation of PKGI increases SSG1 phosphorylation and also induces dissociation of PKGI-SSG1 binding. PKGI activation with either ANP or with the NO donor SNOC induces SSG1 phosphorylation in VSMCs. SSG1 is highly and preferentially expressed in cardiovascular tissues and cells. Finally, we demonstrate that PKGI activation with cGMP regulates SSG1 by inducing its translocation from the membrane to the cytosol in VSMCs. We interpret these findings to support a mechanism in VSMCs in which PKGI binds and phosphorylates SSG1, thereby inducing its cytosolic translocation.

We also observed that, in addition to increasing PKGI phosphorylation of SSG1, cGMP also regulates PKGI-SSG1 interaction by inducing the dissociation of the two proteins. This finding might be explained by a number of models. One possibility is that the cGMP-induced conformational change in PKGI favors termination of its physical association with SSG1. Another possibility is that cGMP induces PKGI to phosphorylate SSG1 at a currently unidentified site, which alters the SSG1 conformation and causes dissociation from PKGI.

NO mediates multiple functions in the vascular wall, including relaxation of the subjacent VSMCs. NO increases intracellular cGMP, which, in turn, activates PKGI. Studies from animal models demonstrate that PKGI itself regulates vascular processes, such as vasorelaxation by phosphorylation of specific substrate proteins. Although multiple PKGI effectors in the VSMC have been characterized, efforts to identify new PKGI substrates have the potential to more fully elucidate the mechanisms of PKGI regulation of VSMC function. From our phosphorylation screen described above, we have now identified SSG1 as a novel PKGI substrate and binding protein in VSMCs. Its preferential expression in cardiovascular tissues and regulation by NO and cGMP supports that SSG1 functions as a vascular PKGI effector *in vivo*.

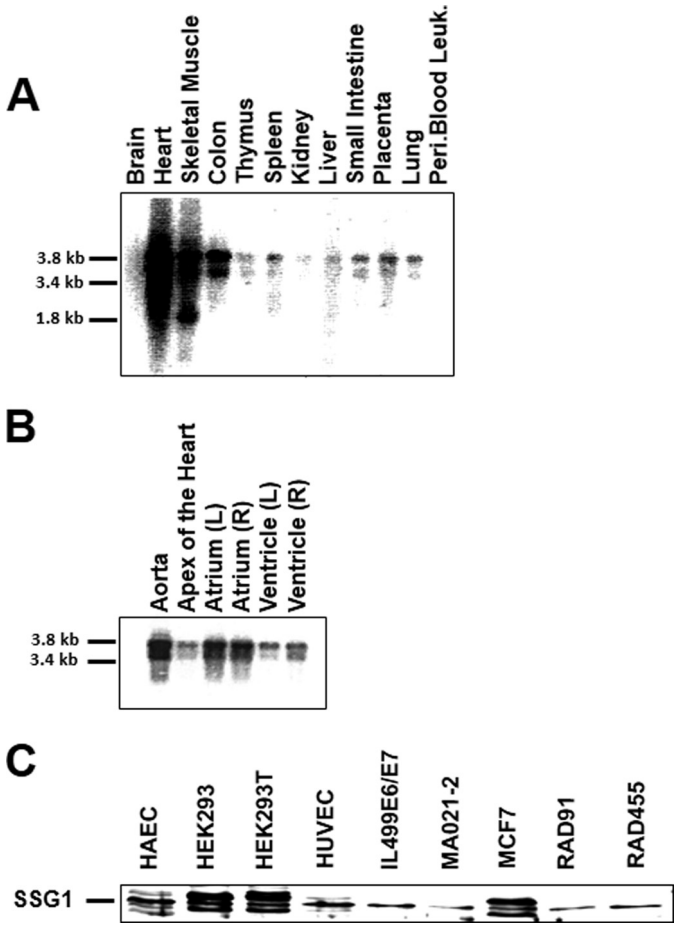

FIGURE 10. **Tissue expression of hSSG1.** *A*, Northern blot analysis of RNA from human tissues. The *arrows* indicate the sizes of the three transcripts identified. *B*, Northern blot analysis of RNA from human cardiovascular tissues. The membranes (Clontech) were probed with ^{32}P-labeled C3-3 DNA followed by autoradiography. *L*, left; *R*, right. *C*, identification of hSSG1 protein in cultured cells. Immunoblot analyses of cell culture lysates using anti-C3-3 IgG. *Peri. Blood Leuk.*, peripheral blood leukocyte.; *HAEC*, human aortic endothelial cell; *HUVEC*, human umbilical vein endothelial cell. Results are representative of three separate experiments.

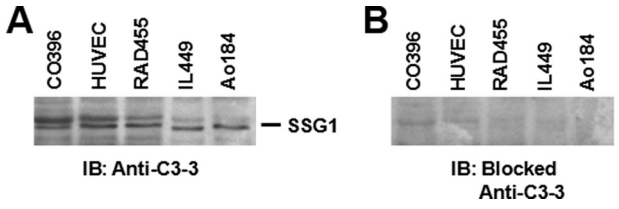

FIGURE 11. **Specificity of rabbit anti-C3-3 (hSSG1) antibody for C3-3.** *A*, detection of C3-3 by Western blot analysis (*IB*) of cell lysates using anti-C3-3 antibody. *HUVEC*, human umbilical vein endothelial cell. *B*, Western blot analysis of identical lysates used in *A* but with coincubation of C3-3-immunizing peptide. Results are representative of three separate experiments.

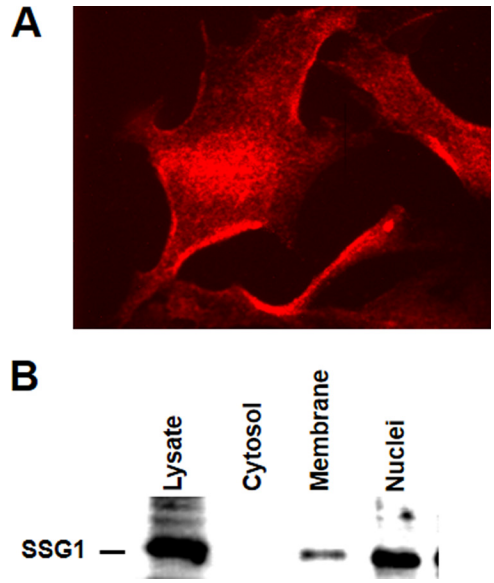

FIGURE 12. **Intracellular localization of hSSG1 in Co396 cells.** *A*, immunofluorescence image of Co396 cells labeled with anti-C3-3 IgG antibody and Cy3-linked anti-rabbit secondary antibody. *B*, subcellular fractionation of Co396 cells by differential centrifugation. Immunoblot analysis of total cell lysate, cytosol, plasma membrane, and nuclear fractions with anti-C3-3 IgG. Results are representative of three separate experiments.

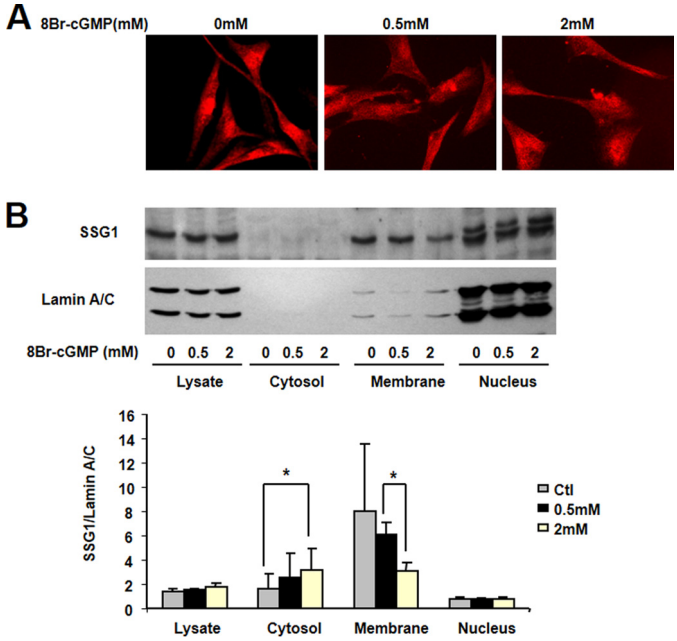

FIGURE 13. **Localization of hSSG1 in Co396 cells treated with 8Br-cGMP.** *A*, immunofluorescence labeling of Co396 cells with anti-C3-3 antibody in the absence and presence of 8Br-cGMP. The concentrations of 8Br-cGMP used are shown above each image. *B*, subcellular fractionation of SSG1 in Co396 cells without and with treatment with 8Br-cGMP. The immunoblot analysis for hSSG1, with anti-C3-3 antibody, and for lamin A/C are shown at the top. The *graph* shows the density of hSSG1 normalized to lamin A/C for each fraction and at each concentration of 8Br-cGMP. $n = 3$. *, $p < 0.05$. *Ctl*, control.

SSG1 was originally cloned in rat tissues as a 42 kDa estrogen-regulated protein (30). SSG1 has also been detected in prostate smooth muscle, where its expression was augmented by androgens (32). The hSSG1 cloned in our study shares homology with rat SSG1 but with important differences. Although both the rat and human SSG1 transcripts are 3.8 kb, R-SSG1 encodes a protein of 42 kDa and hSSG1 encodes a protein of 62 kDa. The open reading frames of the two cDNAs are consistent with their observed molecular weights, with rat SSG1 consisting of 385 amino acids and hSSG1 consisting of 557 amino acids. Interestingly, hSSG1 is identical to the amino-terminal 530 amino acids of the 950 amino acid protein URB (also known as coiled-coil domain containing 80). Both hSSG1 and URB map to the same location of chromosome 3, suggesting that they are transcribed from the same gene. Finally,

immunoblotting of cardiovascular cell lysates using anti-C3-3 antibody reveals a single dominant band corresponding to the predicted size of hSSG1, suggesting that the 62-kDa hSSG1 is the predominant protein expressed in cardiovascular cells. Further studies will be needed to address the relationship between hSSG1 and URB.

We detected the highest expression of SSG1 in heart, skeletal muscle, and the vasculature. This high level of expression of hSSG1 in blood vessels further supports that SSG1 functions as a physiologic PKGI effector. Additionally, the cGMP-induced translocation of SSG1 to the cytosol suggests one mechanism through which PKGI may regulate the as yet unknown vascular function of SSG1. Other cardiovascular PKGI kinase targets, including RhoA (26) and RGS2 (19), also translocate in response to cGMP and PKGI. This shared mechanism with other PKGI kinase targets argues further for the biologic significance of the PKGI-SSG1 interaction.

Although this study identified SSG1 from a VSMC library and focused on its PKGI-dependent regulation in this cell type, it is also notable that hSSG1 expresses highly in the heart. PKGI and cGMP inhibit left ventricular remodeling in response to pressure overload (33, 34), although the downstream PKGI effectors in the myocardium remain incompletely understood. Interestingly, left ventricular pressure overload modulates cardiac SSG1 expression (35). This raises the interesting possibility that the PKGI also regulates SSG1 in the heart, although this admittedly was not tested in this study. It is interesting to note that although cGMP signaling is important in multiple tissues, such as lung, and intestine, we detected the highest SSG1 mRNA expression within vascular and heart tissue. We interpret this observation to suggest a selective role of SSG1 in the regulation of cardiovascular function.

Our study has several limitations. First, it did not specifically test whether PKGI interacts with SSG1 in an isoform-specific fashion. Second, although we provide evidence *in vitro* and in cultured cells that PKGI phosphorylates SSG1 at serines 409 and 434, it remains possible that PKGI phosphorylates SSG1 at sites other than those abolished in our site mutation experiments, such as Ser-79 or Thr-204. Mass spectroscopy analysis of the full-length, intact protein will be required to definitively identify all of the PKGI phosphorylation sites on SSG1. Finally, although we demonstrated ANP- and NO-induced phosphorylation of SSG1 in VSMCs, we did not specifically determine the processes regulated by SSG1 in vascular tissue *in vivo*. Future studies will, therefore, explore the *in vivo* role of SSG1 in modulating cardiovascular function.

In summary, we have identified hSSG1 as a previously unknown PKGI substrate and binding protein in human cardiovascular tissues. The intracellular distribution of hSSG1 is regulated by cGMP, a critical regulator of VSMC contractility and gene expression. These findings, therefore, support that hSSG1 serves as a novel cGMP signaling effector in the vasculature and have the potential to improve the understanding of human cardiovascular physiology and pathophysiology.

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